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Proteolytic Mapping of the Coronavirus Infectious Bronchitis Virus 1b Polyprotein: Evidence for the Presence of Four Cleavage Sites of the 3C-like Proteinase and Identification of Two Novel Cleavage Products

D. X. Liu,[1] S. Shen, H. Y. Xu, and S. F. Wang

Institute of Molecular Agrobiology, The National University of Singapore, 1 Research Link, Singapore 117604

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We have previously reported that the 3C-like proteinase of the coronavirus infectious bronchitis virus (IBV) is responsible for processing of the 1a and 1a/1b polyproteins to three mature products of 24, 10, and 100 kDa (Liu *et al.,* 1994, 1997; Ng and Liu, 1998). The C-terminal cleavage site of the 100-kDa protein was defined to be the $Q^{891(1b)}$-$S^{892(1b)}$ dipeptide bond encoded by nucleotides 15,129 to 15,134 (Liu and Brown, 1995). In this report, other cleavage sites of the 3C-like proteinase in the polyprotein encoded by the ORF 1b region were mapped by coexpression, deletion, and site-directed mutagenesis studies. Using two ORF 1b-specific antisera, V58 and V17, three more Q-S(G) dipeptide bonds, encoded by nucleotides 16,929 to 16,934, 18,492 to 18,497, and 19,506 to 19,511, respectively, were demonstrated to be the cleavage sites of the 3C-like proteinase. Cleavage at these four positions would result in the release of four mature products with molecular masses of approximately 68, 58, 39, and 35 kDa. Among them, the 39- and 35-kDa proteins were specifically identified in IBV-infected cells. Taken together with the 100-kDa protein previously identified, these results suggest that the ORF 1b region of IBV mRNA1 may be able to encode five mature products.

INTRODUCTION

Coronavirus gene expression involves the production of six to seven mRNA species. The prototype species of the Coronaviridae, avian infectious bronchitis virus (IBV), encodes six mRNA species in virus-infected cells. These include the genome-length mRNA (mRNA1) of 27.6 kilobases (kb) and five subgenomic mRNA species (mRNAs 2–6) with sizes ranging from 2 to 7 kb. The available evidence demonstrates that the four virion structural proteins, spike (S), membrane (M), nucleocapsid (N), and small envelope (E), are encoded by subgenomic mRNA 2, mRNA 4, mRNA 6, and the third ORF of mRNA 3, respectively (Liu and Inglis, 1991; Stern and Sefton, 1984). Nucleotide sequencing of the genomic RNA of IBV has shown that the 5′-terminal unique region of mRNA 1 contains two large ORFs (1a and 1b), with ORF 1a having the potential to encode a polyprotein of 441 kDa (1a polyprotein) and 1b having the potential to encode a polyprotein of 300 kDa (Boursnell *et al.,* 1987) (Fig. 1). The downstream ORF 1b is produced as a fusion protein of 741 kDa with 1a (1a/1b polyprotein) by a ribosomal frameshift (Brierley *et al.,* 1987, 1989). The 1a and 1a/1b polyproteins are expected to be cleaved by viral or cellular proteinases to produce functional products associated with viral RNA replication (Fig. 1).

Three proteinase domains have been predicted to be involved in cleavage of the 1a and 1a/1b polyproteins. These include two overlapping papain-like proteinase domains encoded by ORF 1a from nucleotide 4242 to 5553 and one serine proteinase domain of the picornavirus 3C proteinase group (3C-like proteinase) encoded between nucleotides 8937 and 9357 (Gorbalenya *et al.,* 1989; Lee *et al.,* 1991) (Fig. 1). The first papain-like proteinase domain has been demonstrated to be involved in processing of the 1a polyprotein to an 87-kDa mature viral product (Liu *et al.,* 1995; Lim and Liu, 1998). A more important role in the processing of the 1a and 1a/1b polyproteins may be played by the 3C-like proteinase domain, as more than 10 Q-S(G) dipeptide bonds have been predicted to be the cleavage sites of this proteinase (Gorbalenya *et al.,* 1989). Indeed, three mature viral products of 24, 10, and 100 kDa have been demonstrated to be released from the 1a and 1a/1b polyproteins by the 3C-like proteinase-mediated proteolysis (Liu *et al.,* 1994, 1997; Liu and Brown, 1995; Ng and Liu, 1998). Characterization of the 3C-like proteinase demonstrates that the catalytic center of this proteinase is composed of the predicted nucleophilic cysteine residue (Cys^{2922}) and the histidine 2820 (His^{2820}) residue (Liu and Brown, 1995). Meanwhile, *in vitro* expression data suggest that this proteinase may be released from the 1a polyprotein as a 35-kDa protein by autoprocessing at two previously predicted Q-S (Q^{2779}-S^{2780} and Q^{3086}-S^{3087}) dipeptide bonds (Tibbles *et al.,* 1996) (see Fig. 1).

[1] To whom reprint requests should be addressed. E-mail: liudx@ima.org.sg.

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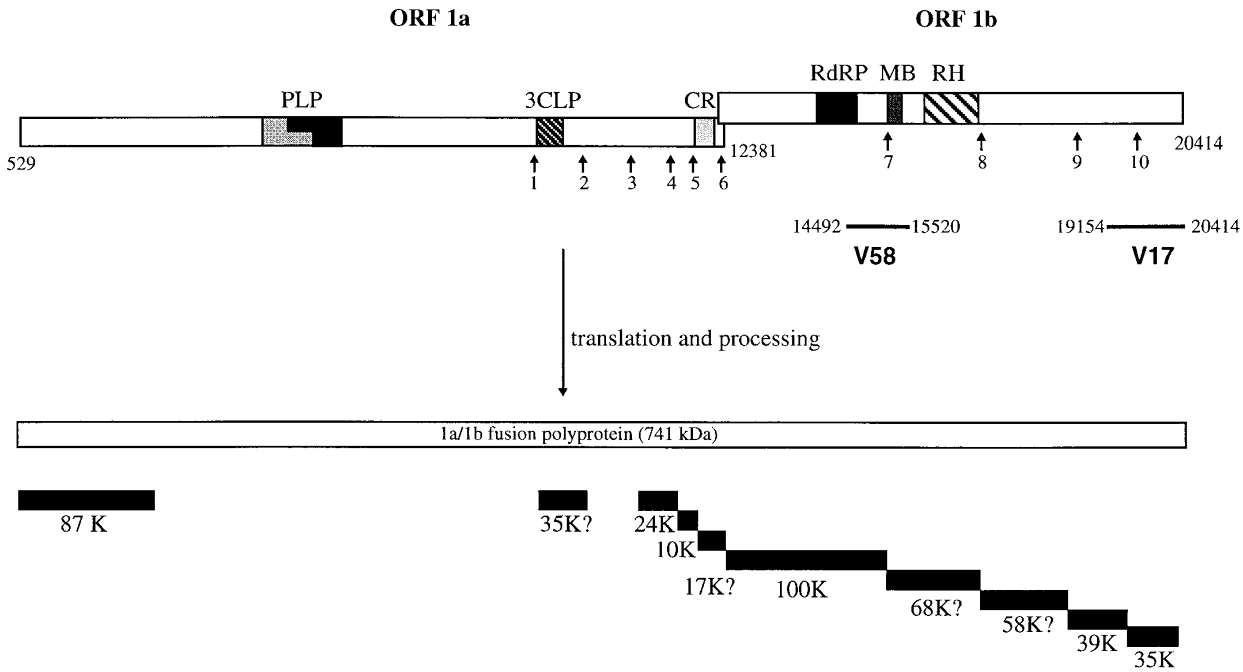

FIG. 1. Diagram showing the structure of ORF 1a and 1b and proteolytic processing map of the 1a/1b polyprotein. The locations of putative functional domains are shown. PLP, papain-like proteinase; 3CLP, 3C-like proteinase; CR, cysteine-rich region; RdRP, RNA-dependent RNA polymerase; MB, metal-binding domain; RH, RNA helicase. Also shown are the regions used to raise antisera V58 and V17 and the positions of 10 identified Q-S(N, G) cleavage sites of the 3C-like proteinase. The precise amino acid positions of the 10 cleavage sites and the nucleotides encoding these dipeptide bonds (given in parentheses) are as follows: 1, Q^{2779}-S^{2780} (8863–8868); 2, Q^{3086}-S^{3087} (9784–9789); 3, Q^{3462}-S^{3463} (10,912–10,917); 4, Q^{3472}-N^{3473} (11,542–11,547); 5, Q^{3783}-S^{3784} (11,875–11,880); 6, Q^{3928}-S^{3929} (12,310–12,315); 7, $Q^{891(1b)}$-$S^{892(1b)}$ (15,129–15,134); 8, $Q^{1491(1b)}$-$S^{1492(1b)}$ (16,929–16,934); 9, $Q^{2012(1b)}$-$S^{2013(1b)}$ (18,492–18,497); 10, $Q^{2350(1b)}$-$S^{2351(1b)}$ (19,506–19,511). The first amino acid position of polyprotein encoded by ORF 1b was counted from the methionine residue encoded by nucleotides 12,459 to 12,461.

Identification and characterization of cleavage products from the 1a and 1a/1b polyproteins have also been reported for human coronavirus and mouse hepatitis virus. In the case of human coronavirus, four mature products have been identified. These include a 34-kDa protein representing the 3C-like proteinase, a 105-kDa protein corresponding to the 100-kDa protein of IBV, a 71-kDa protein with an ATPase activity, and a 41-kDa protein processed from the 1b polyprotein (Ziebuhr *et al.,* 1995; Grotzinger *et al.,* 1996; Heusipp *et al.,* 1997a,b). Similarly, three mature products, including two N-terminal cleavage products of 28 and 65 kDa and a 27-kDa protein identified as the 3C-like proteinase, have been reported for mouse hepatitis virus (Denison and Perlman, 1987; Denison *et al.,* 1995; Lu *et al.,* 1995).

In this communication, we report experiments designed to map the cleavage sites of the 3C-like proteinase in the polyprotein encoded by ORF 1b. Two ORF 1b-specific antisera, V58 and V17 (Fig. 1), were used in this study. Cotransfection, deletion, and site-directed mutagenesis studies demonstrated that three more Q-S(G) cleavage sites, encoded by nucleotides 16929 to 16934, 18492 to 18497, and 19506 to 19511, respectively, were recognized and cleaved by the 3C-like proteinase. Taken together with the two cleavage sites identified for releasing the 100-kDa protein, cleavage at these positions would result in the release of five mature products with molecular masses of approximately 100, 68, 58, 39, and 35 kDa (Fig. 1). Among them, the 100-, 39-, and 35-kDa proteins were specifically identified in IBV-infected cells. Information gained from this study could be used to guide further identification and characterization of the ORF 1b-specific products.

RESULTS

Mutational analysis of the putative $Q^{1491(1b)}$-$G^{1492(1b)}$ cleavage site

We have previously reported the identification of a 100-kDa protein encoded by nucleotides 12,313 to 15,131 in IBV-infected Vero cells using an ORF 1b-specific antiserum V58 (Liu *et al.,* 1994; Liu and Brown, 1995). Two Q-S dipeptide bonds (Q^{3928}-S^{3929} and $Q^{891(1b)}$-$S^{892(1b)}$) encoded by nucleotides 12,310 to 12,315 and 15,129 to 15,134, respectively, were determined to be the N- and C-terminal cleavage sites of the 100-kDa protein. In addition to the $Q^{891(1b)}$-$S^{892(1b)}$ dipeptide bond, three more potential cleavage sites of the 3C-like proteinase have been predicted to be located in the 1b polyprotein (Fig. 1). The first one is the $Q^{1491(1b)}$-$G^{1492(1b)}$ dipeptide bond

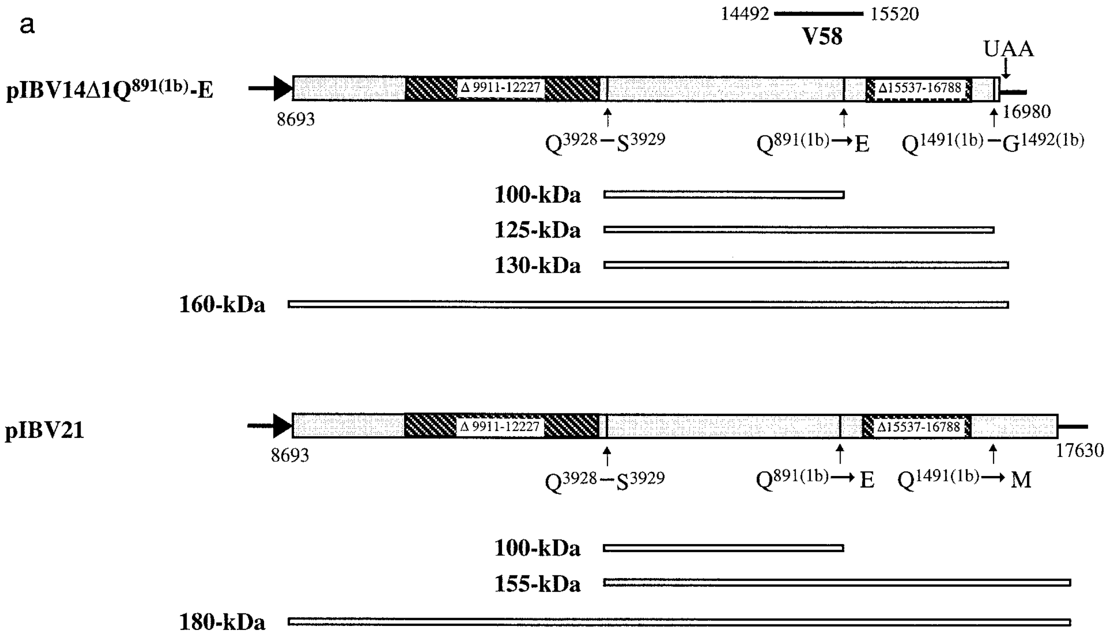

FIG. 2. (a) Diagram showing the IBV sequences present in plasmids pIBV14Δ1Q$^{891(1b)}$-E and pIBV21. The IBV sequence used to raise antiserum V58 and the substitution mutations introduced are indicated. Also shown are the predicted cleavage products that could be detected by immunoprecipitation with antiserum V58 and a UAA termination codon located 47 amino acid residues downstream of the G$^{1492(1b)}$ residue. (b) Analysis of transiently expressed products from transfection of plasmids pIBV14Δ1Q$^{891(1b)}$-E and pIBV21. Plasmid DNAs were expressed in Vero cells using the vaccinia virus-T7 expression system (Fuerst *et al.,* 1986). Semiconfluent monolayers of Vero cells were infected with a recombinant vaccinia virus (vTF7-3) and transfected with plasmid DNA using DOTAP according to the instructions of the manufacturer (Boehringer Mannheim). Cells were labeled with [^{35}S]methionine, lysates were prepared, and polypeptides were either analyzed directly (lanes 1–3) or immunoprecipitated with antiserum V58 (lanes 4–6). The radiolabeled polypeptides were separated on an SDS–12.5% polyacrylamide gel and detected by fluorography. Control DNA (lanes 1 and 4)-a plasmid contains the IBV sequence from nucleotides 8693 to 13,896. HMW, high molecular weight markers (numbers indicate kilodaltons).

encoded by nucleotides 16,929 to 16,934 (Fig. 1). In order to test if this is a genuine scissile bond of the 3C-like proteinase, plasmid pIBV14Δ1Q$^{891(1b)}$-E (Fig. 2a), which was constructed for studying the C-terminal cleavage site of the 100-kDa protein (see Liu and Brown, 1995), was first expressed in Vero cells. As the Q$^{891(1b)}$-E mutation introduced in this construct significantly reduced the cleavage efficiency occurring at the Q$^{891(1b)}$-S$^{892(1b)}$ dipeptide bond, it would allow us to use antiserum V58, which recognizes the 100-kDa protein only, to detect a fusion product containing the 100-kDa protein and products encoded by the downstream region (see Fig. 2a). As expected, expression of pIBV14Δ1Q$^{891(1b)}$-E led to the detection of four polypeptides by immunoprecipitation with antiserum V58 (Fig. 2b, lane 6). In addition to the 100-kDa cleavage product and a 160-kDa protein representing the full-length product expressed from this construct, a 125-kDa protein and a product migrating slightly more slowly on SDS–PAGE than the 125-kDa product were observed (Fig. 2b, lane 6). The estimated molecular weight of the latter species is approximately 130 kDa (Fig. 2b, lane 6). The apparent molecular masses of these two products and their expression patterns suggest that the C-termini of the 125- and 130-kDa proteins may be derived, respectively, from cleavage occurring at the Q$^{1491(1b)}$-G$^{1492(1b)}$ dipeptide bond and from termination of translation at a UAA termination codon 47 amino acid residues downstream of the G$^{1492(1b)}$ residue (see Fig. 2a). It is therefore likely that cleavage may occur at the Q$^{1491(1b)}$-G$^{1492(1b)}$ dipeptide bond.

To support this possibility further, plasmid pIBV21 was constructed and expressed. This plasmid was based on pIBV14Δ1Q$^{891(1b)}$-E, but the Q$^{1491(1b)}$ residue was mutated to an M by PCR (Fig. 2a). Meanwhile, the 3′ end of the IBV sequence present in pIBV14Δ1Q$^{891(1b)}$-E was extended up to nucleotide 17,630 (Fig. 2a). As can be seen, expression of pIBV21 resulted in the detection of three products (Fig. 2b, lane 5). In addition to the 100-kDa protein and the 180-kDa full-length product, a protein species migrating on SDS–PAGE much more slowly than the 125-kDa protein was observed (Fig. 2b, lane 5). Its apparent molecular weight of approximately 155 kDa suggested that it is a fusion protein comprising the 125-kDa protein and the product encoded by nucleotides 16,932 to 17,630 (Fig. 2a). These results confirm that mutation of the Q$^{1491(1b)}$ residue to an M completely blocked cleavage occurring at this position and suggest that the Q$^{1491(1b)}$-G$^{1492(1b)}$ dipeptide bond is a cleavage site of the 3C-like proteinase.

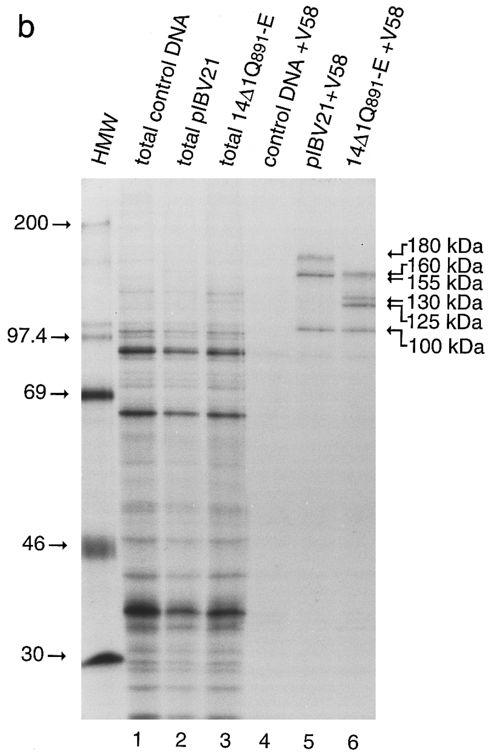

FIG. 2—*Continued*

Proteolytic processing of products encoded by the 3′-terminal 3.5-kb region of ORF 1b

We next set up to analyze if the two predicted cleavage sites ($Q^{2012(1b)}$-$S^{2013(1b)}$ and $Q^{2350(1b)}$-$S^{2351(1b)}$) encoded by nucleotides 18,492 to 18,497 and 19,506 to 19,511, respectively, are cleavage sites of the 3C-like proteinase (see Fig. 1). For this purpose, plasmid pIBV1b4, which covers the IBV sequence from nucleotides 16,932 to 20,490 (Fig. 3a) was expressed either on its own or together with pIBV3C, a plasmid which contains the IBV sequence coding for the 3C-like proteinase and has been shown to possess *trans*-cleavage activity (Liu *et al.,* 1997). As the results in Fig. 3b show, expression of pIBV1b4 in Vero cells led to the synthesis of a protein with an apparent molecular mass of approximately 132 kDa, representing the full-length product encoded by this construct; this product was immunoprecipitated by antiserum V17 (Fig. 3b, lane 1). No processing of the 132-kDa protein to smaller products was observed (Fig. 3b, lane 1). Cotransfection of pIBV1b4 with pIBV3C, however, resulted in the detection of five protein species (Fig. 3b, lane 2). In addition to the 132-kDa full-length product, four other protein species with apparent molecular masses of approximately 97, 74, 39, and 35 kDa, respectively, were also detected (Fig. 3b, lane 2). Among them, the 132-, 97-, 74-, and 39-kDa products were immunoprecipitated efficiently by antiserum V17; only trace amount of the 35-kDa protein, however, was detected from the same cell lysate (Fig. 3b, lane 2). These results suggest that the four smaller products may represent mature and intermediate cleavage products of the 132-kDa polyprotein.

Pulse–chase and time course experiments were then carried out to define further the processing pattern of the products expressed from cotransfection of pIBV1b4 and pIBV3C. For this purpose, Vero cells were cotransfected with pIBV1b4 and pIBV3C, labeled with [^{35}S]methionine for 4 h at 13 h posttransfection, and chased with a 10-fold excess of cold methionine up to 10 h. As shown in Fig. 3c, the 132-, 97-, and 74-kDa proteins were detected after labeling for 2 h and remained detectable after chase for 10 h. The 39- and 35-kDa proteins appeared after chase for 2 h and were increased gradually with time (Fig. 3c).

Further analysis of the expression and processing of products encoded by the 3′-terminal region of ORF 1b

As shown in Fig. 3a, the 132-kDa protein encoded by pIBV1b4 contains two putative Q-S cleavage sites ($Q^{2012(1b)}$-$S^{2013(1b)}$ and $Q^{2350(1b)}$-$S^{2351(1b)}$). Cleavage at these two positions would result in the formation of three mature products with calculated molecular masses of 58, 38, and 34 kDa, respectively. The apparent molecular masses of 39 and 35 kDa of the two proteins detected from coexpression of pIBV1b4 and pIBV3C suggest that they may represent the two C-terminal cleavage products. To investigate this possibility, plasmid pIBV1b5 was constructed and expressed in Vero cells. This plasmid covers the IBV sequence from nucleotides 18,930 to 20,874 and has the potential to encode a product of 59 kDa (Fig. 4a). Transfection of pIBV1b5 alone led to the synthesis of a polypeptide of approximately 59 kDa, consistent with the calculated full-length product of 59 kDa encoded by this construct (Fig. 4b, lane 1). As expected, two additional products with apparent molecular masses of 24 and 35 kDa were produced from coexpression of pIBV1b5 and pIBV3C (Fig. 4b, lane 2). The 35-kDa protein comigrates on SDS–PAGE with the 35-kDa protein produced from coexpression of pIBV1b4 and pIBV3C (Fig. 4b, lanes 2 and 4), indicating that they are the same cleavage product from the C-terminal region of the 1b polyprotein. However, the detection of the 35-kDa protein from coexpression of pIBV1b5 and pIBV3C was disproportionately more efficient than that from coexpression of pIBV1b4 and pIBV3C (Fig. 4b, lanes 2 and 4). The reason for this discrepancy is currently uncertain.

Mutational analysis of the putative $Q^{2350(1b)}$-$S^{2351(1b)}$ and $Q^{2012(1b)}$-$S^{2013(1b)}$ cleavage sites

The results presented above suggest that cleavage of the 1b polyprotein by the 3C-like proteinase may occur at the $Q^{2350(1b)}$-$S^{2351(1b)}$ dipeptide bond, resulting in the release of a mature product of 35 kDa. To support further if cleavage did occur at this position, substitution of the $Q^{2350(1b)}$ residue with an E was introduced by site-

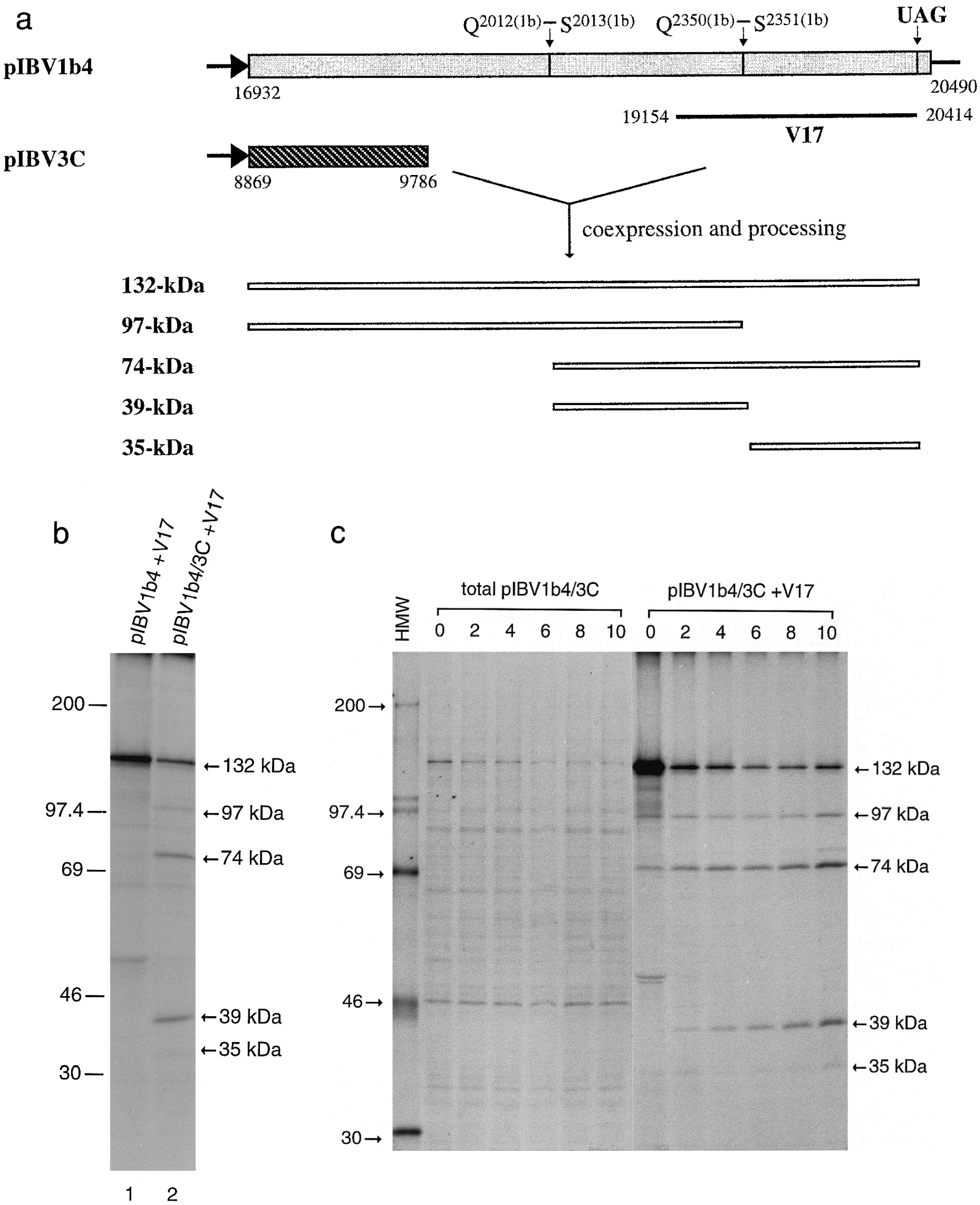

FIG. 3. (a) Diagram showing the IBV sequences present in plasmids pIBV1b4 and pIBV3C. The UAG termination codon for ORF 1b (encoded by nucleotides 20,415 to 20,417) and the IBV sequence used to raise antiserum V17 are indicated. Also shown are the two predicated Q-S ($Q^{2012(1b)}$-$S^{2013(1b)}$ and $Q^{2350(1b)}$-$S^{2351(1b)}$) dipeptide bonds and the predicted cleavage products that could be detected by immunoprecipitation with antiserum V17. (b) Analysis of transiently expressed products from transfection of pIBV1b4 and cotransfection of pIBV1b4 and pIBV3C. Cells were labeled with [^{35}S]methionine, lysates were prepared, and polypeptides were immunoprecipitated with antiserum V17. The radiolabeled polypeptides were separated on an SDS–12.5% polyacrylamide gel and detected by fluorography. Numbers indicate molecular mass in kilodaltons. (c) Pulse–chase analysis of the expression, processing, and accumulation of products from cotransfection of pIBV1b4 and pIBV3C. Cells were labeled with [^{35}S]methionine for 4 h at 13 h posttransfection, chased with a 10-fold excess of cold methionine, and harvested after chase for 0, 2, 4, 6, 8, and 10 h. Cell lysates were then prepared and were either analyzed directly or subjected to immunoprecipitation with antiserum V17. The radiolabeled polypeptides were separated on an SDS–12.5% polyacrylamide gel and detected by fluorography. HMW, high molecular weight markers (numbers indicate kilodaltons).

directed mutagenesis, giving a mutant construct pIBV1b4$Q^{2350(1b)}$-E. Expression of this plasmid in Vero cells resulted in the synthesis, once again, of the full-length 132-kDa polyprotein (Fig. 5, lane 1). Coexpression of pIBV1b4$Q^{2350(1b)}$-E with pIBV3C led to the detection of an additional protein of approximately 74 kDa (Fig. 5, lanes 2 and 5). This product comigrates on SDS–PAGE with the 74-kDa protein detected from cotransfection of pIBV1b4 and pIBV3C (Fig. 5, lanes 5 and 6). No other cleavage products were detected (Fig. 5, lanes 1 and 5),

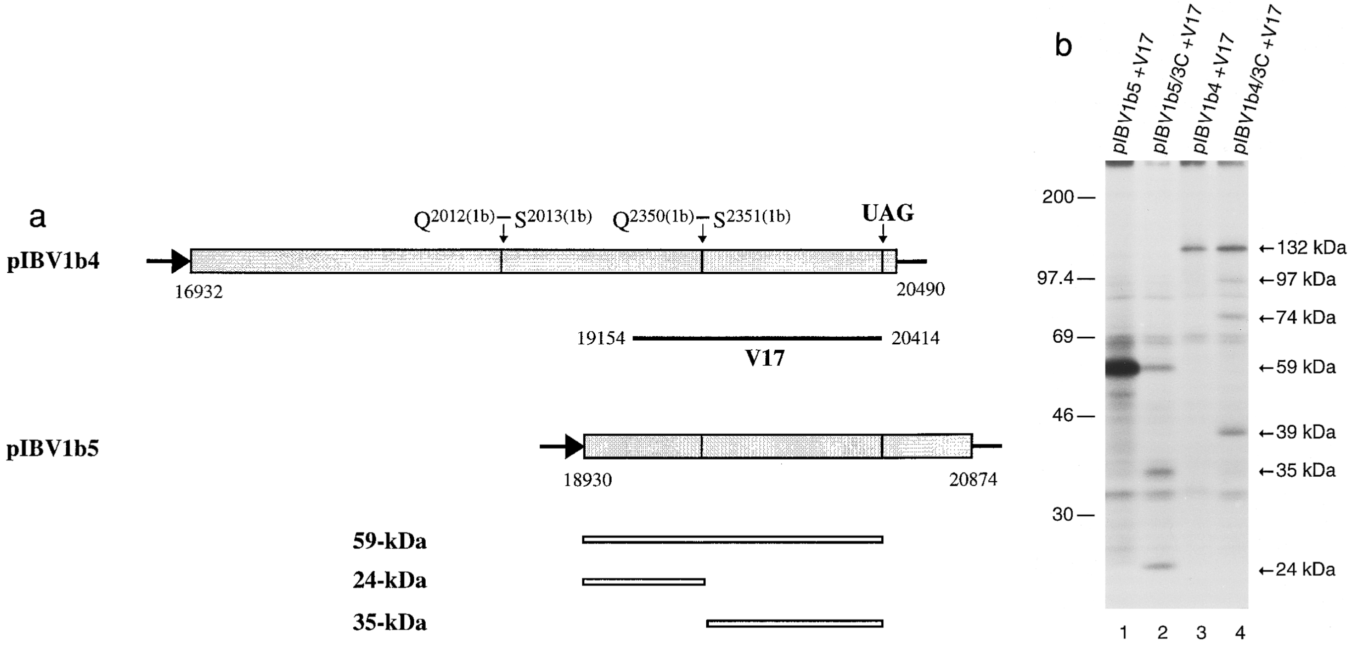

FIG. 4. (a) Diagram showing the IBV sequence present in plasmids pIBV1b4 and pIBV1b5. The UAG termination codon for ORF 1b (encoded by nucleotides 20,415 to 20,417) and the IBV sequence used to raise antiserum V17 are indicated. Also shown are the two predicated Q-S ($Q^{2012(1b)}$-$S^{2013(1b)}$ and $Q^{2350(1b)}$-$S^{2351(1b)}$) dipeptide bonds and the predicted cleavage products that could be detected by immunoprecipitation with antiserum V17. (b) Analysis of transiently expressed products from cotransfection of pIBV1b5 and pIBV3C. Cells were labeled with [^{35}S]methionine, lysates were prepared, and polypeptides were immunoprecipitated with antiserum V17. The radiolabeled polypeptides were separated on an SDS–12.5% polyacrylamide gel and detected by fluorography. Numbers indicate molecular mass in kilodaltons.

suggesting that mutation of the $Q^{2350(1b)}$ residue to an E did block the cleavage occurring at this position. This result indicates that the $Q^{2350(1b)}$-$S^{2351(1b)}$ dipeptide bond may be the cleavage site responsible for releasing the N-terminus of the 35-kDa protein and the C-terminus of the 39-kDa protein from the 1b polyprotein. The 74-kDa protein detected from coexpression of pIBV1b4 and pIBV3C is therefore representing an intermediate cleavage product encoded by ORF 1b from nucleotides 18,495 to 20,414.

Substitution of the $Q^{2012(1b)}$ residue of the predicted $Q^{2012(1b)}$-$S^{2013(1b)}$ cleavage site with an E was subsequently made by site-directed mutagenesis, giving a mutant construct pIBV1b4$Q^{2012(1b)}$-E. Coexpression of this plasmid with pIBV3C led to the synthesis of three protein species, the 132-kDa protein and two products comigrated, respectively, with the 97- and 35-kDa proteins detected from coexpression of pIBV1b4 and pIBV3C (Fig. 5, lanes 4 and 6). No 39- and 74-kDa proteins were detected (Fig. 5, lane 4), suggesting that mutation of the $Q^{2012(1b)}$ residue to an E abolished cleavage occurring at the $Q^{2012(1b)}$-$S^{2013(1b)}$ bond. This result indicates that the $Q^{2012(1b)}$-$S^{2013(1b)}$ dipeptide bond is recognized and cleaved by the 3C-like proteinase to release the N-terminus of the 39-kDa protein and the C-terminus of a putative 58-kDa protein. The 97-kDa protein detected from coexpression of pIBV1b4 and pIBV3C is therefore an intermediate cleavage product containing the 39- and 58-kDa proteins.

Identification of novel gene 1 products encoded by the ORF 1b region in IBV-infected Vero cells

Data presented above indicate that the 35- and 39-kDa proteins may represent mature viral products processed from the C-terminal region of the 1b polyprotein. To confirm this possibility, confluent monolayers of Vero cells were infected with IBV at a multiplicity of infection (m.o.i.) of approximately 2 PFU per cell and were labeled for 4 h with [^{35}S]methionine at 6 h postinfection. Cell lysates were prepared and subjected to immunoprecipitation with antisera V58 and V17. As can be seen, antiserum V58 precipitated specifically, once again, the 100-kDa protein from IBV-infected cell lysates (Fig. 6, lane 3) (also see Liu *et al.,* 1994, 1997). Immunoprecipitation of the same cell lysate with antiserum V17 led to the detection of two polypeptides with apparent molecular masses of 39 and 35 kDa, respectively; no other intermediate cleavage products were detected (Fig. 6, lane 4). Once again, very weak immunoprecipitation of the 35-kDa protein was observed (Fig. 6, lane 4). These two products comigrated on SDS–PAGE with the 39- and 35-kDa proteins detected from coexpression of pIBV1b4 and pIBV3C (data not shown).

DISCUSSION

Coronavirus IBV encodes a proteinase belonging to the picornavirus 3C proteinase group. The available evidence suggests that this proteinase may play a major

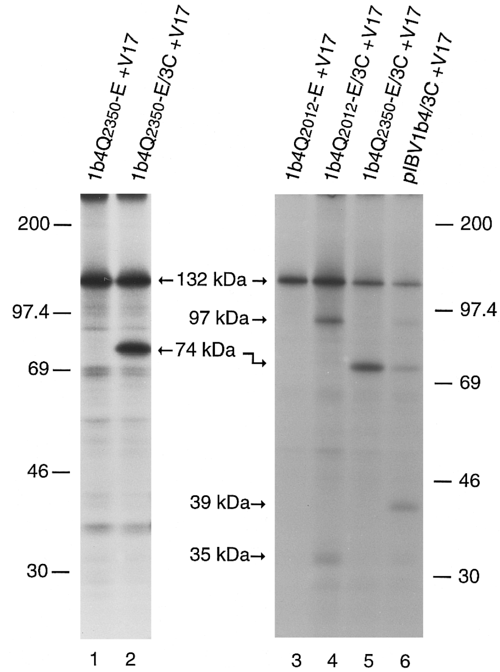

FIG. 5. Mutational analysis of the predicted $Q^{2350(1b)}S^{2351(1b)}$ and $Q^{2012(1b)}S^{2013(1b)}$ cleavage sites. The mutants were transiently expressed in Vero cells using the vaccinia virus-T7 expression system. The transfected cells were labeled with [^{35}S]methionine, lysates were prepared, and polypeptides were immunoprecipitated with antiserum V17. The radiolabeled polypeptides were separated on an SDS–12.5% polyacrylamide gel and detected by fluorography. Numbers indicate molecular mass in kilodaltons.

role in processing of the 1a and 1a/1b polyproteins into mature viral products associated with viral RNA replication. In a previous report, we showed that a 100-kDa mature viral protein was released from the 1a/1b polyprotein by the 3C-like proteinase at two Q-S dipeptide bonds (Q^{3928}-S^{3929} and $Q^{891(1b)}$-$S^{892(1b)}$) (Liu *et al.,* 1994; Liu and Brown, 1995) (see Fig. 1). In this report, we demonstrate that the 3C-like proteinase may also mediate cleavage at other three predicted Q-S(G) dipeptide bonds ($Q^{1491(1b)}$-$G^{1492(1b)}$, $Q^{2012(1b)}$-$S^{2013(1b)}$ and $Q^{2350(1b)}$-$S^{2351(1b)}$) located in the 1b region of the 1a/1b polyprotein (see Fig. 1). In addition to the 100-kDa protein, four mature viral products with calculated molecular masses of approximately 68, 58, 38, and 34 kDa would be released from the 1a/1b polyprotein by cleavage at these positions (see Fig. 1). Indeed, two polypeptides of 39 and 35 kDa, corresponding to the two C-terminal cleavage products of the 1a/1b polyprotein, were identified in IBV-infected Vero cells.

Attempts have been made to detect the rest two cleavage products (the putative 68- and 58-kDa proteins) in virus-infected cells. Mutagenesis studies presented in this report strongly suggest that the two predicted cleavage sites ($Q^{1491(1b)}$-$G^{1492(1b)}$ and $Q^{2012(1b)}$-$S^{2013(1b)}$) flanking the putative 58-kDa protein are genuine cleavage sites of the 3C-like proteinase. The expression and processing patterns shown also lend support to the genuine presence of the 58-kDa protein. In order to detect this product, a region-specific antiserum was raised in rabbits and was shown to be able to precipitate specifically the *in vitro* synthesized target products (data not shown). However, further application of this antiserum in identification of the putative 58-kDa protein from IBV-infected cells was hampered by the fact that it can cross-react with a cellular protein migrating on SDS–PAGE at approximately the same position as the 58-kDa protein (Liu *et al.,* unpublished observations). Two antisera against the putative 68-kDa metal-binding and helicase protein have been raised in rabbits. Both antisera can efficiently recognize the *in vitro* synthesized target proteins. However, it was consistently found that no specific processed product can be detected in cells either infected with IBV or transfected with plasmids covering the region. As the equivalent protein has recently been identified in cells infected with human coronavirus (Heusipp *et al.,* 1997a), it is likely that the 68-kDa protein is a genuine viral product. We are currently uncertain if the failure to detect this product is due to its rapid degradation in intact cells. Systematic analysis of the expression and accumulation of this putative protein is underway to address this possibility.

As both the putative 68- and 58-kDa proteins are currently undetectable in intact cells and expression of

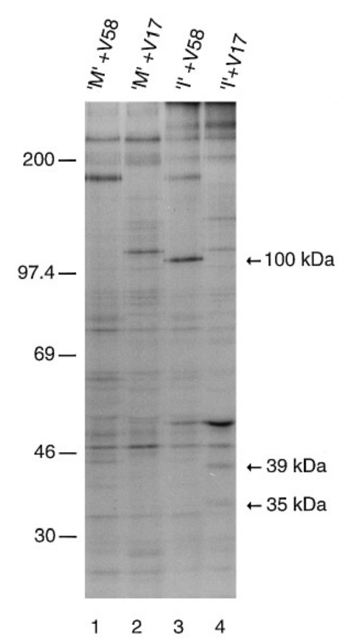

FIG. 6. Detection of a 39- and a 35-kDa protein in IBV-infected Vero cells. Confluent monolayers of Vero cells were infected with IBV at a m.o.i. of 2 PFU per cell. The IBV-infected ('I') and mock-infected ('M') cells were labeled with [^{35}S]methionine, lysates were prepared, and polypeptides were immunoprecipitated with antisera V58 (lanes 1 and 3) and V17 (lanes 2 and 4). The radiolabeled polypeptides were separated on an SDS–12.5% polyacrylamide gel and detected by fluorography. Numbers indicate molecular mass in kilodaltons.

the region encoding the putative 68-kDa protein is elusive, the previously predicted $Q^{1491(1b)}$-$G^{1492(1b)}$ cleavage site was studied by using region-specific antiserum V58. For the same reason, plasmid pIBV14Δ1$Q^{891(1b)}$-E, a construct containing two deletions and a mutation of the $Q^{891(1b)}$ residue to an E, was expressed to study the expression and processing pattern of the regions flanking this site. The results generated as well as the mutagenesis studies reported here suggest that the $Q^{1491(1b)}$-$G^{1492(1b)}$ dipeptide bond is a real scissile bond of the 3C-like proteinase. The rest two predicted cleavage sites ($Q^{2012(1b)}$-$S^{2013(1b)}$ and $Q^{2350(1b)}$-$S^{2351(1b)}$) were studied based on the *trans*-cleavage activity of the 3C-like proteinase. Once again, data generated from expression, deletion and mutagenesis studies indicate that they are genuine cleavage sites of the 3C-like proteinase. However, coexpression, pulse-chase and time course experiments presented in this study showed that cleavage at these two positions is much less efficient than other cleavage sites identified (Liu and Brown, 1995; Liu *et al.,* 1997). As can be seen in Fig. 3c, the 97-kDa and 74-kDa intermediate cleavage products remained detectable even after chase for 10 h, suggesting that these two Q-S dipeptide bonds may be poor cleavage sites of the 3C-like proteinase. However, no intermediate cleavage products could be detected in virus-infected cells (Fig. 6). This may reflect some regulatory mechanisms controlling the balance between the production and accumulation of the proteinase and its substrate in virus-infected cells. In IBV-infected cells, the 3C-like proteinase is produced approximately five times as much as the 1a/1b polyprotein, as the 1a/1b polyprotein is expressed by ribosome frameshifting and the frameshifting efficiency is 25–30% (Brierley *et al.,* 1987, 1989). Approximately equal amounts of the proteinase and substrate, however, would be expected to be produced in a single cell by coexpression of two plasmids. It is therefore understandable that complete cleavage of the 1a/1b polyprotein can be achieved in virus-infected cells by an excess amount of the proteinase. Nevertheless, it would be of interest to define the sequence determinants that dictate the cleavage efficiency of a particular substrate.

Currently, no functional domain has been found in the regions encoding the 39- and 35-kDa proteins. A counterpart of the IBV 39-kDa protein, the human coronavirus 41-kDa protein, has recently been shown to exhibit a punctate, perinuclear distribution in virus-infected cells (Heusipp *et al.,* 1997b), indicating that it may be located in the membranous compartment. It would be of interest to see if the 39-kDa protein shows a similar cellular distribution to the 41-kDa protein. This possibility as well as the significance of such a distribution in viral RNA replication is currently under investigation.

MATERIALS AND METHODS

Virus and cells

The egg-adapted Beaudette strain of IBV (ATCC VR-22) was obtained from the American Type Culture Collection (ATCC) and was adapted to Vero cells as described by Alonso-Caplen *et al.* (1984). Briefly, the virus was passaged three times in 11-day-old chicken embryos and then adapted to Vero cells (ATCC CCL-81) by a series of passages at 24- to 48-h intervals. The cytopathic effects, including syncytium formation and rounding up of cells, were initially observed after three passages in Vero cells. Virus stocks were prepared after the 36th passage by infecting monolayers of Vero cells at a m.o.i. of approximately 0.1 PFU/cell. The virus was harvested at 24 h postinfection and the titer of the virus preparation was determined by plaque assay on Vero cells.

Vero cells were grown at 37°C in 5% CO_2 and maintained in Glasgow's modified minimal essential medium (GMEM) supplemented with 10% newborn calf serum.

Labeling of IBV-infected cells with [^{35}S]methionine

Confluent monolayers of Vero cells were infected with IBV at a m.o.i. of approximately 2 PFU/cell. Prior to being labeled, the cells were incubated in methionine-free medium for 30 min. After 4 h of labeling with 25 μCi [^{35}S]methionine, the cells were scraped off the dishes in phosphate-buffered saline, recovered by centrifugation, and stored at −80°C.

Transient expression of IBV sequences in Vero cells using a vaccinia-T7 expression system

Open reading frames placed under control of the T7 promoter were expressed transiently in eukaryotic cells as described previously (Liu *et al.,* 1994). Briefly, semiconfluent monolayers of Vero cells were infected with 10 PFU/cell of a recombinant vaccinia virus (vTF7-3) which expresses the bacteriophage T7 RNA polymerase and then transfected with the appropriate plasmid DNA using DOTAP transfection reagent according to the instructions of the manufacturer (Boehringer Mannheim). After incubation of the cells at 37°C for 4 h, 25 μCi/ml [^{35}S]methionine was added directly to the medium. The radiolabeled cells were harvested at 18 h posttransfection.

Polymerase chain reaction (PCR)

Appropriate primers and template DNAs were used in amplification reactions with Pfu DNA polymerase (Stratagene) under standard buffer conditions with 2 mM $MgCl_2$. PCR conditions were 30 cycles of 95°C for 45 s, X°C for 45 s, and 72°C for X min. The annealing temperature (X°C) and the extension time (X min) were adjusted according to the melting temperature of the primers used and the length of the PCR fragments synthesized.

Site-directed mutagenesis

Site-directed mutagenesis was carried out, as previously described (Liu and Brown, 1995), using single-stranded DNA templates prepared from plasmid pIBV1b4 and appropriate oligonucleotide primers.

Substitution mutation of the $Q^{1491(1b)}$-$G^{1492(1b)}$ dipeptide bond was introduced by two rounds of PCR using two pairs of primers, as previously described (Liu *et al.,* 1997).

Radioimmunoprecipitation

Plasmid DNA-transfected Vero cells were lysed with RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% NP-40) and precleared by centrifugation at 12,000 rpm for 5 min at 4°C in a microfuge. Immunoprecipitation was carried out as described previously (Liu *et al.,* 1994).

SDS–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of virus polypeptides was carried out using 12.5% polyacrylamide gels (Laemmli, 1970). Labeled polypeptides were detected by autoradiography or fluorography of dried gels.

Construction of plasmids

Plasmid pIBV1b4 was made by cloning an *Nco*I- and *Bam*HI-digested PCR fragment into *Nco*I- and *Bam*HI-digested pKTO (Liu *et al.,* 1994). The PCR fragment covers the IBV sequence from nucleotides 16,932 to 20,490 and contains an artificial AUG initiation codon in an optimal context (ACC<u>AUG</u>G) located immediately upstream of the viral sequence. The sequence of the upstream primer used to generate this PCR fragment was 5′-ACAAGTCCC<u>ATG</u>GGTACAGGTT-3′, and the downstream primer was 5′-GCACCCCCGGGATCCTGCCAAC-3′. Plasmid pIBV1b5 was constructed by cloning an *Sna*BI- and *Dra*I-digested fragment containing the IBV sequence from nucleotides 18,930 to 20,874 into *Eco*RV- and *Sma*I-digested pKTO and selected by nucleotide sequencing.

Two mutants with alterations at the putative $Q^{2350(1b)}$-$S^{2351(1b)}$ and $Q^{2012(1b)}$-$S^{2013(1b)}$ cleavage sites were made by site-directed mutagenesis using single-stranded DNA templates prepared from plasmid pIBV1b4. Plasmid pIBV1b4$Q^{2350(1b)}$-E was made by site-directed mutagenesis using oligonucleotide primer 5′-TCCACAGCTTGAATCAGCATG-3′, and pIBV1b4$Q^{2012(1b)}$-E was made by using oligonucleotide primer 5′-TTCAGCTCTCGAGTCTATCGAC-3′.

Plasmid pIBV14Δ1$Q^{891(1b)}$-E, which contains the $Q^{891(1b)}$ to an E mutation (Liu and Brown, 1995), was used to construct plasmid pIBV21 as follows. An 842-bp PCR fragment, which covers the IBV sequence from nucleotides 16,788 to 17,630 and contains a $Q^{1491(1b)}$ to $M^{1491(1b)}$ mutation, was generated by PCR. This PCR fragment was then digested with *Pst*I, gel-purified, and ligated into *Pst*I- and *Sma*I-digested pIBV14Δ1$Q^{891(1b)}$-E, giving plasmid pIBV21. The sequence of the oligonucleotide primer used to introduce the mutation was 5′-ACAAGTCCCATGGGTACAGGTT-3′, and the sequence of the complementary primer was 5′-AACAAACCTGTACCCATGGGAC-3′. This construct was selected by restriction digestion with *Nco*I and confirmed by automated nucleotide sequencing.

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